APPLICATION

FOR

UNITED STATES PATENT

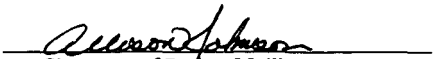
TITLE: **METHOD OF MEASURING MOLECULAR INTERACTIONS**

APPLICANT: **DAVID E. WOLF; DYLAN BULSECO**

Certificate of Express Mailing

Pursuant to 37 CFR 1.10 I certify that this application is being deposited on the date indicated below with the United States Postal Service "Express Mail Post Office to Addressee" service addressed to: Commissioner for Patents, Mail Stop Patent Application, Alexandria, VA 22313-1450.

ET982318591US
Express Mail Mailing Label No. Application


Signature of Person Mailing

August 1, 2003
Date of Deposit

Allison Johnson
Printed Name of Person Mailing Application

METHOD OF MEASURING MOLECULAR INTERACTIONS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application Serial No. 60/461,394, filed April 8, 2003, U.S. Provisional Application Serial No. 60/400,503 filed August 1, 2002, and U.S. Provisional Application Serial No. 60/430,273 filed December 2, 2002.

BACKGROUND

The invention relates to assaying for the interaction of a target and a probe using fluorescence correlation spectroscopy.

Various scientific disciplines related to the biological sciences seek to detect the presence of molecules, understand molecular interactions, and determine the properties and functions of molecules. The Human Genome Project has provided a library of all proteins expressed in the human body. Understanding the molecular interactions of these expressed proteins and evaluating the function of expressed proteins has the potential to lead to the development of new drugs and new drug therapies. Likewise, the threat posed by the potential use of pathogens against populations as a form of biological warfare has highlighted the need to detect pathogens.

Numerous assay techniques have been developed to assist the study of molecular interactions including, e.g., Enzyme-Linked Immunosorbent Assays (ELISA), Radio-Immunoassays (RIA), fluorescence assays, dynamic light scattering, mass spectrometry, yeast 2-hybrid, phage display, and calorimetric assays. Many of these assay techniques require specialized preparation, purification, separation and amplification of the sample to be tested. Some of these assay techniques also require relatively large amounts of sample and are time consuming to conduct. Accordingly, there exists a need for development of methods of measuring molecular interactions and that are relatively simple to implement and can be conducted on a relatively small amount of sample.

Fluorescence correlation spectroscopy (FCS) is a single molecule detection method that measures the fluctuations in fluorescence intensity in a small (e.g., femtoliter) confocal volume. FCS employs a tightly focused laser beam to define the confocal volume. The diffusion of fluorescently labeled particles into and out of the illuminated volume determines the fluorescence intensity fluctuation patterns. From this data, one can

extract both qualitative information and quantitative information on the molecule being studied. Such qualitative information includes, e.g., the presence or absence of molecular interaction; such quantitative information includes diffusion time, stoichiometry of the interactions, concentration of the interacting particles and the kinetics of the interaction.

FCS has been used to study a variety of properties of single molecules including translational diffusion and transport, chemical kinetics, molecular aggregation, ligand binding, enzymatic activity, and nucleic acid interactions.

SUMMARY

In a first aspect the invention features a method of assaying for the equilibrium interaction of a probe and an unknown target, the method including exciting a sample at with radiation, the sample including at least a portion of the members of a library, at least one probe, and at least one fluorescent tag, measuring the fluorescence from a subvolume of the sample, and analyzing the fluctuations of the fluorescence. In one embodiment the method further includes selecting additional portions of the library, sequentially exciting an additional portion of the library with radiation, measuring the fluorescence of a subvolume of the additional portion, and analyzing the fluctuations of the fluorescence. In some embodiments, the sample includes a plurality of fluorescent tags, the fluorescent tags being attached to the members.

In one embodiment the method further includes separating at least one of the members of the portion of the library from at least one other member of the portion of the library, and repeating the method on the at least one separated member.

In some embodiments, the members include the fluorescent tag. In other embodiments the fluorescent tag is attached to the probe.

In another embodiment the method further includes generating a library. In one embodiment the method includes generating a library that includes fluorescent members. In some embodiments the generating includes in vitro translation. In other embodiments the method further includes labeling the members of the library with a fluorophore. In some embodiments the labeling includes in vitro translation labeling using a fluorescent amino acid analogue, labeling by inserting a sequence for a fluorescent protein into a cDNA or post translational labeling.

In some embodiments the members of the library include fluorescent proteins. In other embodiments the members of the library include fluorescently tagged amino acids. In other embodiments the members of the library include fluorescently labeled peptides.

In one embodiment the sample includes a plurality of unique probes, each unique probe including a unique fluorescent tag, each unique probe having a unique binding site.

In other embodiments when binding of a probe and a member is present, the method further includes identifying the member with which the probe has formed a bond.

the sample further includes a second fluorescently labeled probe, the first fluorescently labeled probe and the second fluorescently labeled probe being capable of binding to two different unique binding sites.

In one embodiment the sample further includes a second probe capable of binding to a unique site on a target, the unique site being created when the first probe binds to the target.

In other embodiments the at least one fluorescent tag is attached to a second probe, the second probe being capable of binding to a unique site on at least one of a target and the first probe when the first probe is bound to the target, the unique site being created when the first probe binds to the target. In some embodiments the unique site is derived from a change in at least one of the primary, secondary and tertiary structure of at least one of the target and the first probe. In another embodiment the unique site is created by the addition of a moiety to the target. In other embodiments the unique site is created by at least one of phosphorylation, glycosylation, alkylation, acylation, acetylation, and ubiquitination. the unique site is created by proteolysis. In other embodiments the unique site is selected from the group consisting of a phosphotyrosine, phosphoserine, or a combination thereof.

In one embodiment the members include a binding site created by at least one of phosphorylation, glycosylation, proteolysis, and ubiquitination.

In some embodiments at least one of the probe and the member is attached to a bead. In other embodiments the probe is attached to the bead and the fluorescent tag is attached to the member. In another embodiment the member is attached to the bead and the fluorescent tag is attached to the probe.

In one embodiment the analyzing includes determining at least one of the size of the fluorescence intensity fluctuations and the duration of the correlation of the fluorescence fluctuation. In other embodiments the analyzing includes determining a correlation function including at least one of the crosscorrelation function of the sample and an autocorrelation function of the sample. In another embodiment the analyzing further includes determining the decay time of the correlation function. In some embodiments the analyzing further includes determining the time zero value of the correlation function. In other embodiments the analyzing includes at least one of a moment analysis, Fourier transform analysis, and a power spectrum analysis.

In one embodiment when binding is present, the method further including determining at least one of the diffusion coefficient of a probe-member complex, the number of probe-member complexes in the sample, and the stoichiometry of the probe-member complex.

In some embodiments the sample further includes a plurality of unique probes, wherein each unique probe includes a unique fluorophore. In other embodiments the sample further includes a plurality of different size beads, a plurality of probes and a plurality of members of the library, at least one of the probes and the members being attached to the beads. In one embodiment the members are attached to the beads and the probes include a fluorescent tag. In other embodiment the probes are attached to the beads and the members include a fluorescent tag.

In other embodiments the sample further includes a second fluorescent tag different from the first fluorescent tag. In other embodiments the first fluorescent tag is attached to the probe and the second fluorescent tag is attached to the member. In some embodiments the first fluorescent tag is attached to the first probe and the second fluorescent tag is attached to at least one of a second probe and a bead. In another embodiment the first fluorescent tag is attached to the member and the second fluorescent tag is attached to at least one of the probe and a bead.

In one embodiment the sample further includes a plurality of different size beads and at least one of the probe and the member is attached to the beads. In other embodiments the sample further includes a plurality of unique probes, each unique probe being attached to a different size bead. In some embodiments the first fluorescent tag is

attached to the probe and the second fluorescent tag is attached to the unknown target. In other embodiments first fluorescent tag is attached to the first probe and the second fluorescent tag is attached to at least one of a second probe and the beads.

In another embodiment the sample includes a crosslinking agent. In some embodiments at least one of the probe, the member, and the fluorescent tag includes a crosslinking agent.

In another embodiments the method further includes obtaining at least one of a true autocorrelation function and a true crosscorrelation function of the sample.

In some embodiments further includes flowing the sample through a sample chamber. In other embodiments the method is automated.

In some embodiments the method further includes determining the true autocorrelation function (G_{1T}) of the fluorescence of the sample measured at a first detector of a fluorescence correlation spectroscopy instrument having a first detector, a second detector and an excitation source, using the following equation or its equivalent

$$G_{1T} = \frac{-2\rho < I_1 >< I_2 > R + \rho^2 < I_2 >^2 G_2 + < I_1 >^2 G_1}{-2\rho < I_1 >< I_2 > + \rho^2 < I_2 >^2 + < I_1 >^2}$$

where G_{1T} is the true autocorrelation function of the fluorescence measured at the first detector, ρ is the bleed through coefficient of the second detector into the first detector, $<I_1>$ is the time averaged intensity in the first detector, $<I_2>$ is the time averaged intensity in the second detector, R is a measured crosscorrelation function between the first detector and the second detector, and G_1 and G_2 are measured autocorrelation functions of the first detector and the second detector, respectively.

In other embodiments the method further includes determining the true autocorrelation function (G_{2T}) of the fluorescence of the sample measured at a second detector of a fluorescence correlation spectroscopy instrument having a first detector, a second detector and an excitation source, using the following equation or its equivalent

$$G_{2T} = \frac{-2r < I_1 >< I_2 > R + < I_2 >^2 G_2 + r^2 < I_1 >^2 G_1}{-2r < I_1 >< I_2 > + < I_2 >^2 + r^2 < I_1 >^2}$$

where G_{2T} is the true autocorrelation function of the fluorescence measured at the second detector, r is the bleed through of the first detector into the second detector and $<I_1>$, $<I_2>$, R, G_1 and G_2 are as described above.

In one embodiment, the method further includes determining the true crosscorrelation function (R_T) of the fluorescence of the sample measured at a first detector of a fluorescence correlation spectroscopy instrument having a first detector, a second detector and an excitation source, using the following equation or its equivalent

$$R_T = \frac{< I_1 >< I_2 > R(1+\rho r) - \rho < I_2 >^2 G_2 - r < I_1 >^2 G_1}{< I_1 >< I_2 > (1+\rho r) - \rho < I_2 >^2 - r < I_1 >^2}$$

where R_T is the true crosscorrelation function of the fluorescence measured at the first and second detectors, $<I_1>$, $<I_2>$, R, G_1 and G_2 are as described above.

In another aspect, the invention features a method of assaying for the equilibrium interaction of a probe and an unknown target, the method including exciting a sample with radiation, the sample including at least one unknown target, at least one probe, and at least one fluorescent tag, measuring the fluorescence from a subvolume of the sample, and analyzing the fluctuations of the fluorescence. In some embodiments the fluorescent tag is attached to the unknown target. In other embodiments when binding is present, the method further includes identifying the unknown target with which the probe has formed a bond.

In some embodiments the unknown target includes a product resulting from pathogen infection. In other embodiments the unknown target includes a toxin.

In another aspect, the invention features a method of assaying for a pathogen in a sample, the method including exciting a sample with radiation, the sample including a pathogen, at least one probe, and at least one fluorescent tag, measuring the fluorescence from a subvolume of the sample, and analyzing the fluctuations of the fluorescence.

In a second aspect, the invention features a method of assaying for the presence of a pathogen component in a sample, the method including exciting a sample with radiation, the sample including at least one probe capable of binding a predetermined pathogen component, and at least one fluorescent tag, measuring the fluorescence from a subvolume of the sample, analyzing the fluctuations of the fluorescence, and determining the presence or absence of the pathogen component. In some embodiments the method further includes identifying the pathogen.

In one embodiment the pathogen component includes a bacterium. In other embodiments the pathogen component includes a virus. In another embodiment the

pathogen component is selected from the group consisting of pathogen, pathogen fragment, pathogen nucleic acid, pathogen protein, pathogen carbohydrate, and combinations thereof. In some embodiments the pathogen component is selected from the group consisting of pathogen spore, pathogen toxin, metabolic product of pathogen, and combinations thereof. In other embodiments the pathogen component is a pathogen and the probe is capable of binding to a pathogen.

In some embodiments the sample includes a plurality of unique fluorescently tagged probes, each unique probe including a unique fluorophore, each unique probe being capable of binding to a unique pathogen component.

In another embodiment the analyzing includes determining at least one of a crosscorrelation function of the sample and determining an autocorrelation function of the sample.

In a third aspect, the invention features a method of assaying for the presence of a toxin in a sample, the method including exciting a sample with radiation, the sample including at least one probe capable of binding a predetermined toxin, and at least one fluorescent tag, measuring the fluorescence from a subvolume of the sample, analyzing the fluctuations of the fluorescence, and determining the presence or absence of the toxin. In one embodiment the toxin is ricin. In some embodiments the probe and the fluorescent tag include fluorescently tagged human serum albumin galactose.

In a fourth aspect, the invention features a method of identifying a probe capable of binding to a known pathogen, the method including exciting a sample with radiation, the sample including at least one known pathogen, at least one probe, and at least one fluorescent tag, measuring the fluorescence emitted by the sample, and analyzing the fluctuations of the fluorescence.

In a fifth aspect, the invention features a kit including a first probe including ricin, a fluorescent tag attached to the ricin, and a second probe bound to the first probe, the second probe being adapted to bind ricin. In some embodiments the second probe includes human serum albumin galactose. In other embodiments the kit further includes a second fluorescent tag. In one embodiment the second fluorescent tag is attached to the second probe.

In a sixth aspect, the invention features a method of assaying for the presence of molecular interactions of a probe and a target, the method including exciting a sample with radiation, the sample including a plurality of unique mass adding components each unique mass adding component having a unique mass, a plurality of targets, a plurality of fluorescent tags, and a plurality of probes, and measuring the fluorescence emitted by the sample, and analyzing the fluctuations of the fluorescence. In some embodiments, the fluorescent tags are attached to the mass adding component. In other embodiments, the fluorescent tags are attached to the probes. In some embodiments, the fluorescent tags are attached to the targets. In another embodiment, the probes are attached to the mass-adding component. In one embodiment the fluorescent tags are attached to the probes and the probes are attached to the mass adding component. In another embodiment, the fluorescent tags are attached to the mass adding component and the probes are attached to the mass adding component. In some embodiments, the plurality of fluorescent tags include a plurality of unique fluorescent tags.

In one embodiment, the kit includes a plurality of unique beads, each unique bead having a different size, a plurality of probes adapted to bind to a unique target, the probes being attached to the beads, and a plurality of fluorescent tags. In one embodiment, the fluorescent tags include unique fluorophores. In other embodiments the kit further includes a second probe. In some embodiments, the fluorescent tags are attached to at least one of the beads and the probes.

In a seventh aspect, the invention features a method of determining a true correlation function of a sample, the method including obtaining a measured correlation function of the sample from a fluorescence correlation spectroscopy instrument and applying a correction algorithm to the measured correlation function. In one embodiment, the spectroscopy instrument includes an excitation source, a first detector, and a second detector. In some embodiments the measured correlation function is an autocorrelation function. In other embodiments the measured correlation function is a crosscorrelation function. In one embodiment, the correction algorithm adjusts the measured correlation function based on a bleed through coefficient.

In other embodiments the correction algorithm is further based on a first average of the fluorescence intensities measured at the first detector and a second average of the fluorescence intensities measured at the second detector.

In one embodiment, the invention features a method of determining a true autocorrelation function of a sample, the method including obtaining a first measured autocorrelation function (G_1) of the sample from a first detector of a fluorescence correlation spectroscopy instrument, obtaining a second measured autocorrelation function (G_2) of the sample from a second detector of the instrument, obtaining a measured crosscorrelation function (R) between the first detector and the second detector of the instrument, calculating a first time averaged intensity (I_1) of the fluorescence at the first detector, calculating a second time averaged intensity (I_2) of the fluorescence at the second detector, determining the true autocorrelation function (G_{1T}) of the fluorescence measured at the first detector using the following equation or its equivalent

$$G_{1T} = \frac{-2\rho < I_1 >< I_2 > R + \rho^2 < I_2 >^2 G_2 + < I_1 >^2 G_1}{-2\rho < I_1 >< I_2 > + \rho^2 < I_2 >^2 + < I_1 >^2}$$

where ρ is a bleed-through coefficient of the second detector into the first detector.

In another embodiment, the method of determining a true autocorrelation function of a sample includes obtaining a first measured autocorrelation function (G_1) of the sample from a first detector of a fluorescence correlation spectroscopy instrument, obtaining a second measured autocorrelation function (G_2) of the sample from a second detector of the instrument, obtaining a measured crosscorrelation function (R) between the first detector and the second detector of the instrument, calculating a first time averaged intensity (I_1) of the fluorescence at the first detector, calculating a second time averaged intensity (I_2) of the fluorescence at the second detector, determining a true autocorrelation function (G_{2T}) of the fluorescence measured at the second detector using the following equation or its equivalent

$$_{t}\, G_{2T} = \frac{-2r < I_1 >< I_2 > R + < I_2 >^2 G_2 + r^2 < I_1 >^2 G_1}{-2r < I_1>< I_2 > + < I_2 >^2 + r^2 < I_1 >^2}$$

where r is a bleed-through coefficient of first detector into the second detector.

In other embodiments, the method of determining a true crosscorrelation function of a sample includes obtaining a first measured correlation function (G_1) of the sample

from a first detector of a fluorescence correlation spectroscopy instrument, obtaining a second measured correlation function (G_2) of the sample from a second detector of the instrument, obtaining a measured crosscorrelation function (R) between the first detector and the second detector of the instrument, calculating a first time averaged intensity (I_1) of the fluorescence at the first detector, calculating a second time averaged intensity (I_2) of the fluorescence at the second detector, determining a true crosscorrelation function (R_T) using the following equation or its equivalent

$$R_T = \frac{< I_1 >< I_2 > R(1+\rho r) - \rho < I_2 >^2 G_2 - r < I_1 >^2 G_1}{< I_1 >< I_2 > (1+\rho r) - \rho < I_2 >^2 - r < I_1 >^2}$$

where ρ is a bleed-through coefficient of the second detector into the first detector, and r is a bleed-through coefficient of the first detector into the second detector.

In an eighth aspect, the invention features an article of manufacture that includes a computer readable medium having stored therein a computer program for determining a true correlation function of a sample, the computer program including a first code segment for obtaining a measured correlation function of the sample, and a second code segment for applying a correction algorithm to the measured correlation function. In one embodiment, the measured correlation function is an autocorrelation function. In other embodiments, the measured correlation function is a crosscorrelation function. In some embodiments the correction algorithm adjusts the measured correlation function based on a crosstalk parameter between the first and the second detectors. In other embodiments the correction algorithm is further based on a first average of the fluorescence intensities measured at the first detector and a second average of the fluorescence intensities measured at the second detector.

In a ninth aspect the invention features an article of manufacture including a computer readable medium having stored therein a computer program for determining a true correlation function of a sample, the computer program including a first code segment for obtaining a first measured autocorrelation function (G_1) of the sample from a first detector of a fluorescence correlation spectroscopy instrument, a second measured autocorrelation function (G_2) of the sample from a second detector of the instrument, and a measured crosscorrelation function (R) between the first detector and the second detector of the instrument, a second code segment for calculating a first time averaged intensity (I_1)

of the fluorescence at the first detector and a second time averaged intensity (I_2) of the fluorescence at the second detector, a third code segment for determining the true autocorrelation function (G_{1T}) of the fluorescence measured at the first detector using the following equation or its equivalent

$$G_{1T} = \frac{-2\rho < I_1 >< I_2 > R + \rho^2 < I_2 >^2 G_2 + < I_1 >^2 G_1}{-2\rho < I_1 >< I_2 > + \rho^2 < I_2 >^2 + < I_1 >^2}$$

where ρ is a bleed-through coefficient of the second detector into the first detector. In one embodiment, the article further includes a fourth code segment for determining a second true autocorrelation function (G_{2T}) of the fluorescence measured at the second detector using the following equation or its equivalent:

$$G_{2T} = \frac{-2r < I_1 >< I_2 > R + < I_2 >^2 G_2 + r^2 < I_1 >^2 G_1}{-2r < I_1>< I_2 > + < I_2 >^2 + r^2 < I_1 >^2}$$

where r is a bleed-through coefficient of the first detector into the second detector. In some embodiments the article further includes a fifth code segment for determining a true crosscorrelation function (R_T) using the following equation or its equivalent:

$$R_T = \frac{< I_1 >< I_2 > R(1 + \rho r) - \rho < I_2 >^2 G_2 - r < I_1 >^2 G_1}{< I_1 >< I_2 > (1 + \rho r) - \rho < I_2 >^2 - r < I_1 >^2}.$$

In one embodiment, the article of manufacture includes a computer readable medium having stored therein a computer program for determining a true correlation function of a sample, the computer program including a first code segment for obtaining a first measured correlation function (G_1) of the sample from a first detector of a fluorescence correlation spectroscopy instrument, a second measured correlation function (G_2) of the sample from a second detector of the instrument, and a measured crosscorrelation function (R) between the first detector and the second detector of the instrument, a second code segment for calculating a first time averaged intensity (I_1) of the fluorescence at the first detector and a second time averaged intensity (I_2) of the fluorescence at the second detector, a third code segment for determining the true autocorrelation function (G_{2T}) of the fluorescence measured at the second detector using the following equation or its equivalents

$$G_{2T} = \frac{-2r < I_1 >< I_2 > R + < I_2 >^2 G_2 + r^2 < I_1 >^2 G_1}{-2r < I_1 >< I_2> + < I_2 >^2 + r^2 < I_1 >^2}$$

where r is a bleed-through coefficient of the first detector into the second detector.

In another embodiment, the article of manufacture including a computer readable medium having stored therein a computer program for determining a true correlation function of a sample, the computer program including a first code segment for obtaining a first measured correlation function (G_1) of the sample from a first detector of a fluorescence correlation spectroscopy instrument, a second measured correlation function (G_2) of the sample from a second detector of the instrument, and a measured crosscorrelation function (R) between the first detector and the second detector of the instrument, a second code segment for calculating a first time averaged intensity (I_1) of the fluorescence at the first detector and a second time averaged intensity (I_2) of the fluorescence at the second detector, a third code segment for determining the true crosscorrelation function (R_T) using the following equation or its equivalent

$$R_T = \frac{< I_1 >< I_2 > R(1+\rho r) - \rho < I_2 >^2 G_2 - r < I_1 >^2 G_1}{< I_1>< I_2 > (1+\rho r) - \rho < I_2 >^2 - r < I_1 >^2}.$$

where ρ is a bleed-through coefficient of the second detector into the first detector and r is a bleed-through coefficient of the first detector into the second detector.

In a tenth aspect, the invention features a system for determining a true correlation function of a sample, the system including a memory device for storing information related to the sample and a processor programmed with instruction to obtain a measured correlation function of the sample from a fluorescence correlation spectroscopy instrument and apply a correction algorithm to the measured correlation function.

In another aspect, the invention features a fluorescence correlation spectroscopy instrument for determining a true correlation function of a sample, the device including an excitation source, a first detector and a second detector for measuring fluorescence of the sample, a memory device for storing information related to the sample, and a processor programmed with instruction to obtain a measured correlation function of the sample from a fluorescence correlation spectroscopy instrument and apply a correction algorithm to the measured correlation function.

In another embodiment, the method of determining a true fluorescence intensity of a sample includes obtaining a measured fluorescence intensity of the sample from a first detector of a fluorescence correlation spectroscopy and applying a correction algorithm to the measured fluorescence intensity. In one embodiment, the correction algorithm adjusts the measured fluorescence intensity based on a bleed-through coefficient between the first detector and a second detector of a fluorescence correlation spectroscopy. In other embodiments, the correction algorithm is further based on a second measured fluorescence intensity of the sample from the second detector.

In one embodiment, the method of determining a true fluorescence intensity of a sample includes measuring a first fluorescence intensity (I_1) of the sample at a first detector of a fluorescence correlation spectroscopy instrument and a second fluorescence intensity (I_2) of the sample at a second detector of a fluorescence correlation spectroscopy instrument, determining a first true fluorescence intensity (X) of the fluorescence measured at the first detector using the following equation:

$$\boxed{X = \frac{I_1 - \rho I_2}{1 - \rho r}}$$

where ρ is a first bleed-through coefficient of the second detector into the first detector and r is a second bleed-through coefficient of the first detector into the second detector.

In another embodiment, the method of determining a true fluorescence intensity of a sample, the method including measuring a first measured fluorescence intensity (I_1) and a second measured fluorescence intensity (I_2) of the sample from the first and second detectors, respectively, determining the true fluorescence intensity fluorescence intensity (Y) of the fluorescence measured at the second detector using the following equation

$$\boxed{Y = \frac{I_2 - rI_1}{1 - \rho r}}$$

where ρ is a first bleed-through coefficient of the second detector into the first detector and r is a second bleed-through coefficient of the first detector into the second detector.

In one embodiment, the method further includes generating a true autocorrelation curve, based on the first true fluorescence intensity.

In another embodiment, the method further includes generating a true crosscorrelation curve, based on the first and second true fluorescence intensities.

In one embodiment, the article of manufacture includes a computer readable medium having stored therein a computer program for determining a true fluorescence intensity of a sample, the computer program including a first code segment for obtaining a first bleed-through coefficient (ρ) of a second fluorescence spectroscopy detector into a first fluorescence spectroscopy detector and a second bleed-through coefficient (r) of the first detector into the second detector, a second code segment for measuring a first measured fluorescence intensity (I_1) and a second measure fluorescence intensity (I_2) of the sample from the first and second detectors, respectively, a third code segment for determining a first true fluorescence intensity (X) of the fluorescence measured at the first detector using the following equation:

$$\boxed{X = \frac{I_1 - \rho I_2}{1 - \rho r}}.$$

In other embodiments, the article of manufacture includes a computer readable medium having stored therein a computer program for determining a true fluorescence intensity of a sample, the computer program including a first code segment for obtaining a first bleed-through coefficient (ρ) of a second fluorescence spectroscopy detector into a first fluorescence spectroscopy detector and a second bleed-through coefficient (r) of the first detector into the second detector, a second code segment for measuring a first measured fluorescence intensity (I_1) and a second measure fluorescence intensity (I_2) of the sample from the first and second detectors, respectively, and a third code segment for determining a true fluorescence intensity (Y) of the fluorescence measured at the second detector using the following equation

$$\boxed{Y = \frac{I_2 - rI_1}{1 - \rho r}}.$$

In another embodiment, the fluorescence correlation spectroscopy instrument for determining a true fluorescence intensity of a sample includes an excitation source, a first detector and a second detector for detecting a first measured fluorescence (I_1) and a second

measured fluorescence (I_2) of the sample, a memory device for storing computer code, and a processor for executing the computer code to obtain the true fluorescence intensity, based on the first and the second measured fluorescence.

In one embodiment, the computer code includes instructions for determining a true fluorescence intensity (X) of the fluorescence measured at the first detector using the following equation:

$$X = \frac{I_1 - \rho I_2}{1 - \rho r}$$

where ρ is a bleed-through coefficient of detector two into detector one and r is a bleed-through coefficient of detector one into detector two.

In some embodiments, the computer code includes instructions for determining a true fluorescence intensity (Y) of the fluorescence measured at the second detector using the following equation:

$$Y = \frac{I_2 - rI_1}{1 - \rho r}.$$

where ρ is a bleed-through coefficient of detector two into detector one and r is a bleed-through coefficient of detector one into detector two.

In one embodiment the memory device is an EPROM.

The invention features a method for studying macromolecular interactions such as protein-protein, protein-DNA, protein-RNA, DNA-DNA, RNA-DNA, and RNA-RNA interactions using fluorescence correlation spectroscopy. The method can be used to determine the stoichiometric nature of a molecule (e.g., the number of binding sites on a molecule), the molecular mass of a molecule, the number of fluorescent particles in a sample, and combinations thereof. The method can be used to identify which members of a library bind to a predetermined probe, as well as which probes bind to a predetermined target.

The invention also features the ability to identify novel binding partners to a specific target, e.g., proteins, and to detect the presence and/or determine the identity of a target in a sample containing unknown targets.

The invention also features a method of determining a true autocorrelation function and a true crosscorrelation function.

The invention also features kits including reagents for assaying known and unknown targets.

Other features and advantages will be apparent from the following description of the preferred embodiments and from the claims.

GLOSSARY

In reference to the invention, these terms have the meanings set forth below:

The term "probe" means any known component with a binding site.

The term "fluorescent tag" or "fluorescently tagged" means the presence of a fluorophore and includes fluorophore, fluorophore-containing moieties that are capable of binding to other moieties, and combinations thereof.

The term "assay" means determining the presence or absence of a target, the amount of a target, or both.

The term "library" means a number of related members that differ from each other in some aspect of their chemical structure.

The term "target" means a component to which a binding site of the probe binds.

The term "unknown target" means a component to which it is not known whether or not the probe binds.

The term "particle" means a fluorescent tag.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A illustrates the fluorescence intensity fluctuations over time of Example 1.

FIG. 1B illustrates the crosscorrelation curve of Example 1.

FIG. 2A illustrates the fluorescence intensity fluctuations of Example 2.

FIG. 2B illustrates the crosscorrelation curve corresponding to the data of FIG. 2A.

FIG. 3A illustrates the fluorescence intensity fluctuations of Example 3.

FIG. 3B illustrates the crosscorrelation curve that results from the data in FIG. 3A.

FIG. 4A illustrates fluorescence intensity fluctuations of Example 4.

FIG. 4B illustrates the autocorrelation curve for the data collected in FIG. 4A.

FIG. 5A illustrates the autocorrelation curve of Example 5.

FIG. 5B illustrates the use of the parameter estimates for determining the fraction of slow diffusing particles (F_2Np) at each IgG concentration.

FIG. 5C illustrates the use of parameter estimates from the analysis of the

autocorrelation curve of FIG. 5A to determine stoichiometry.

FIG. 6A illustrates the autocorrelation curves from untreated (solid line) and NGF-treated (dotted line) A875 cells of Example 6.

FIG. 6B illustrates theoretical curves for monomers, dimers, trimers and tetramers as a function of fractional occupancy.

FIG. 7 illustrates the autocorrelation curves of Example 7.

FIG. 8 illustrates the autocorrelation curves of Example 8.

FIG. 9 illustrates the crosscorrelation data collected for Example 9

FIG. 9A illustrates the crosscorrelation data collected for Example 9 before applying the cross-talk correction algorithm.

FIG. 9B illustrates the crosscorrelation data collected for Example 9 after applying the correction algorithm.

FIG. 10A illustrates autocorrelation data for Example 10 before applying the cross-talk correction algorithm

FIG. 10B illustrates autocorrelation data for Example 10 after applying the cross-talk correction algorithm.

FIG. 11 illustrates moment analysis on intensity fluctuation data for Example 11.

FIG. 12A illustrates the power spectrum of a Fourier transform of intensity fluctuation data for Example 12.

FIG. 12B illustrates the amplitude spectrum of a Fourier transform of intensity fluctuation data for Example 12.

DETAILED DESCRIPTION

The present invention provides a method of screening members of a library (e.g., proteins produced from a cDNA library) using fluorescence correlation spectroscopy. The method includes screening a sample that includes a sub-volume of the library, at least one probe and at least one fluorescent tag to determine whether a probe binds to a member of the library under equilibrium conditions. Depending on the results of the screening, the method optionally includes conducting separation and screening processes on the subvolume of the library or additional subvolumes of the library until the identity of a member that binds with the probe can be established. Any suitable method for separating and screening a library to identify the individual members of the library can be used.

The method determines the presence or absence of binding between a member (or members) of the library and a probe (or probes) by analyzing the fluctuations in fluorescence emitted by a subvolume of the sample. Analysis of the measured fluctuations can provide information about various properties of the sample including, e.g., the presence or absence of binding between the probe and a member, the number of binding sites available on a member, diffusion coefficients, diffusion time, number of fluorescently tagged complexes present in the subvolume of the sample, the number of members to which a probe binds in a sample, counts per member, average intensity, aggregation state chemical concentration, chemical reaction kinetics, stoichiometry and combinations thereof. These properties can be determined for members in solution, as well as in the plasma membrane of a living cell.

The method can also be used to assay for molecular interactions between an unknown target and a probe in a sample that includes at least one probe, at least one unknown target and at least one fluorescent tag, and to determine the presence or absence of binding between a probe and an unknown target by analyzing the fluctuations in fluorescence emitted by a subvolume of the sample.

The method also is suitable for a variety of applications including, e.g., analyzing samples thought to contain a pathogen or toxin, screening sterilized samples for infection, continuous monitoring of a sample stream for potential targets, and combinations thereof.

Analysis of the measured fluctuations of a sample can provide the same information about a system that includes an unknown target, pathogen, or toxins as set forth above with respect to members of a library.

I. FLUORESCENCE CORRELATION SPECTROSCOPY (FCS)

The fluorescence of a sample can be measured using an FCS instrument, which generally includes at least one light source, light focusing device adapted to focus light emitted by the light source on a sample, at least one detector capable of detecting light, and a correlator coupled to the detector, the correlator being capable of processing data received at said detector and providing data including autocorrelation data, crosscorrelation data, or a combination thereof. In the case of crosscorrelation, at least two detectors configured to measure around two distinct wavelength maxima are required.

Suitable FCS instruments are described, e.g., in U.S. Patent application Serial No. 60/461,394 entitled, "Fluorescence Correlation Spectroscopy Instrument and Method of Using the Same," and incorporated herein. Other suitable FCS instruments are described, e.g., in Bulseco, D. A. and Wolf, D. E. (2003). "*Fluorescence Correlation Spectroscopy.*" Video Microscopy, Second Edition. Sluder, G. and Wolf, D. Eds. Academic Press, New York.;_Magde, D., E.L. Elson, and W.W. Webb, *Fluorescence correlation spectroscopy. II. An experimental realization.* Biopolymers, 1974. 13(1): p. 29-61.; Rigler, R., *Fluorescence correlations, single molecule detection and large number screening. Applications in biotechnology.* J Biotechnol, 1995. 41(2-3): p. 177-86 and incorporated herein.

The fluorescence measured by the system can be analyzed using various techniques including, e.g., fluorescence correlation spectroscopy in an autocorrelation mode, fluorescence correlation spectroscopy in a crosscorrelation mode, Moments analysis, Fourier transform analysis, which includes power spectrum analysis and amplitude analysis, and combinations thereof.

Fluorescence correlation spectroscopy (FCS) is a technique that is used to extract relevant information from the intensity fluctuations of fluorescent tags that diffuse through or are driven through the confocal volume of an FCS instrument. FCS measures the decay of temporal correlation in fluorescence intensity in the confocal volume. FCS can be run in an autocorrelation mode, a crosscorrelation mode or both modes, sequentially or simultaneously.

Correlation techniques characterize an event by at least two parameters. In the case of fluorescent tags, which are herein sometimes referred to as "particles," correlation data provides the number of independent particles present in the sample and, in the case of diffusion, whether or not the particles are exhibiting a diffusion coefficient (i.e., diffusion time) that is characteristic of the particle or the complex formed by a particle, probe, target and combinations thereof. The relaxation time for correlation relates to stochastic processes of randomization such as the diffusion or the rate of driven flow of targets through the confocal volume, while the size of these fluctuations relates to the number of particles involved in the stochastic process. The binding of two fluorescently tagged

probes on the same target results in an increase in the amplitude (R) of the correlation when FCS is conducted in crosscorrelation mode.

Autocorrelation measures the persistence of a single fluorescent particle in the confocal volume. More specifically, autocorrelation measures the correlation between the intensity of the fluorescence at time $\tau=0$ with all subsequent times. Specific binding of a single probe to a target may result in a change in the diffusion time (τ_D) of the target and probe complex. These changes can be detected using a fluorescence correlation spectroscopy instrument functioning in the autocorrelation mode. Autocorrelation functions also can be used to analyze the fluctuations in fluorescence intensity to yield information on other properties of the particles and targets in the sample including, e.g., aggregation state chemical concentration, chemical reaction kinetics, stoichiometry and combinations thereof. This information can be obtained on targets in solution, as well as in the plasma membrane of living cells.

Autocorrelation measures a change in intensity, $\delta I(t)$, about the average intensity, and a change in intensity, $\delta I(t + \tau)$, around the mean of the intensity of the sample at some time τ later. Statistical analysis of fluorescence intensity fluctuations results in an autocorrelation curve, which shows the decay of temporal correlation in fluorescence intensity over time. The autocorrelation function $G(\tau)$ is given by

$$G(\tau) = 1 + \frac{< \delta I(t) * \delta I(t+\tau) >}{< I >^2}, \qquad (1)$$

where δI refers to the deviation of the intensity about the mean, t is the true time, τ is the incremental time, I is the intensity of the fluctuation, and where $< >$ refers to averaging over all times t.

The value of the autocorrelation function at time $\tau=0$ is the reciprocal of the average number of particles in the sample, and can be used as a measure of complexing or aggregation of the particles.

Crosscorrelation temporally correlates the intensity fluctuations of two different (i.e., unique) fluorophores with distinct excitation and emission properties. Coincidence of these fluorophores on the same macromolecule is detected as a change in amplitude of the crosscorrelation function, R, at short time points, τ, which is directly proportional to the

concentration of dual-tagged fluorescent particles. The crosscorrelation function is given by:

$$r(\tau) = \frac{< \delta I_i(t) * \delta I_j(t+\tau) >}{SD_i * SD_j} \qquad (2)$$

The correlation function of Equation 2 is the form commonly used in statistics. The correlation function goes to 1 for perfect crosscorrelation and to 0 for no crosscorrelation. Instrumentally, it is simpler to define the crosscorrelation function in a manner analogous to Equation 1 for the autocorrelation function R(τ).

$$R(\tau) = 1 + \frac{< \delta I_i(t) * \delta I_j(t+\tau) >}{< I >_i * < I_j >} \qquad (3)$$

where I_i refers to the intensity in channel one, and I_j refers to the intensity in channel two. All other aspects of the notation are the same as those used to describe Equation 1. This form is simpler to calculate in real-time from an ongoing data stream and has the further advantage that when $I_i=I_j$, then $R(\tau) = G(\tau)$.

Equations 1-3 represent the statistical analysis of the fluctuations. Determination of specific molecular properties from these equations requires a knowledge of the physical causes of the fluctuation. If the dependence of the correlation function on these molecular properties is physically modeled, then nonlinear regression can be used to fit the data to the model. For instance, in the case of multiple component three dimensional (3D) solution diffusion coupled with intersystem crossing between fluorescence molecular singlet and triplet states, the autocorrelation function is given by Equation 4.

$$G(\tau) = 1 + \left(\frac{1}{N}\right)\left(1 - T + T\exp\left(-\tau/\tau_T\right)\right)\left(\sum_i \frac{F_i}{\left(1+\tau/\tau_{D_i}\right)\left(1+\tau/K^2\tau_{D_i}\right)^{1/2}}\right) \qquad (4)$$

where N is the number of particles, T is the triplet state fraction, τ_T is the triplet state correlation time, F_i particle fraction, and τ_{Di} diffusion time for diffusing particle species *i*. The structure parameter, K^2 where $K=\omega_2/\omega_1$ (ω_2 and ω_1 being the exp(-2) beam radii in the z and x directions respectively) is determined separately and held constant for each fit.

II. CORRELATION

Various permutations of a target-probe-fluorescent tag system can be used to study the binding properties of a target using fluorescence correlation spectroscopy. For ease of discussion, the following examples of the various embodiments of the system and methods that employ the systems will be described with reference to an unknown target. It is to be understood, however, that the discussion is also applicable to members of a library, known targets, pathogens, toxins and combinations thereof.

A. AUTOCORRELATION

Various permutations of a target-probe-fluorescent tag system can be used to study the binding properties of a target using fluorescence correlation spectroscopy in an autocorrelation mode. The system includes at least one unknown target, at least one probe and at least one fluorescent tag. At least one of the unknown target, the probe, or the complex formed when an unknown target is bound to a probe (i.e., the probe-target complex) includes a fluorescent tag. The components of the system are selected such that the diffusion coefficient of the fluorescently tagged component changes when a molecular interaction, such as probe-target binding, occurs (which is reflected in an increase in the decay time of the correlation function), the particle number (N) changes due to crosslinking, or a combination thereof. Various system configurations are suitable.

In one embodiment, the unknown target(s) of the system includes a fluorescent tag. A probe is added to the system, and, if binding between the probe and a fluorescently tagged unknown target occurs, the diffusion coefficient of the fluorescently tagged unknown target changes. If the probe has multiple binding sites to which the unknown target can bind, the particle number will also change.

In another embodiment, the probe of the system includes a fluorescent tag and, when the fluorescently tagged probe is added to a sample that includes an unknown target, if binding of the probe and an unknown target occurs, the diffusion coefficient of the fluorescently tagged probe changes. If the unknown target has multiple binding sites to which the probe can bind, the particle number will also change.

In another embodiment, a first probe is selected such that binding of the first probe to an unknown target creates a site to which a second probe that includes a fluorescent tag can bind. When the first probe is added to a sample that includes the unknown target, and if binding between the first probe and the unknown target occurs, the second fluorescently tagged probe will bind to the newly created binding site, and the diffusion coefficient of the second fluorescently tagged probe will change. If the unknown target has multiple binding sites to which the second probe can bind, the particle number will also change. If the first probe does not bind to an unknown target, the second probe will not be capable of binding to the unknown target and the diffusion coefficient of the second probe will not change.

The newly created site can be derived from a change in at least one of the primary, secondary and tertiary structure of the unknown target. The new site can also be created by various mechanisms including, e.g., the addition of a moiety to the target, phosphorylation, glycosylation, alkylation, acylation, acetylation, and ubiquitination, and the cleavage of a moiety, e.g., proteolysis. For example, a fluorescently labeled probe can be selected to recognize a specific site on a target created when an enzymatic reaction occurs. The enzymatic reaction causes specific events to occur that create a novel binding site to which the specific probe can bind. Enzymatic reactions can be induced naturally (e.g., in the cell) or after addition of an inducing agent (e.g., in an assay system). Examples of suitable sites that can be created as a result of probe binding include phosphotyrosine, phosphoserine, and combinations thereof, as well as all of the added moieties as described above to specific glycolipid or glycoprotein sites.

If a probe, a fluorescent tag, or an unknown target is bound to a mass-adding component such as a bead, and the components of the system are selected such that a binding event corresponds with the fluorescent tag of the system being associated with the bead, the increased mass of the complex imparted by the bead will cause a change in the diffusion coefficient of the fluorescent tag that is more pronounced relative to the change in the diffusion coefficient in the absence of the bead. If the binding event causes multiple fluorescent tags to bind to a single component, e.g., an unknown target, a bead or a probe, the particle number will also change. For ease of discussion, the mass-adding component will be referred to herein as a bead, however, it is to be understood that any mass-adding

component that does not interfere with the desired molecular interactions of the components of the system can be used. Other suitable mass-adding components include, e.g., crosslinking agents, biotin/avidin complexes, biotin/strepavidin complexes, whole antibody molecules, complexes of whole antibodies, polymeric amino acids, nucleic acids, carbohydrates, specific resins composed of mass adding components, and combinations thereof.

Examples of useful bead components include quantum dots, inactivated bacteria, microspheres of polymers (e.g. polystyrene), alginate, acrylamide, agarose, and sepharose. Suitable beads are commercially available from Molecular Probes (Eugene, OR), Quantum Dot (Hayward, California) and Bangs Laboratories (Fishers, Indiana). Particularly useful beads are available, e.g., under the trade designation PROACTIVE from Bangs Laboratories and under the trade designations QUANTUM-PLEX protein coated microspheres (e.g. coated with streptavidin, protein A, or antibodies) and QDOT Strepavidin and QDOT 655 Protein A Conjugate all of which are available from Quantum Dot.

In one embodiment, the system includes a probe attached to a bead, a number of unknown targets that include a common epitope, and a fluorescent tag. The fluorescent tag is capable of binding to the common epitope. If a probe binds to an unknown target, the diffusion coefficient of the fluorescent tag bound to the epitope will change. If the probe has multiple binding sites to which the unknown target can bind, the particle number will change. If the bead has multiple probes capable of binding the unknown target attached to it, then the particle number will change. The fluorescent tag can be added prior to or subsequent to the binding of an unknown target to a probe.

In other embodiments, the method includes a competitive assay in which the sample includes two probes, one of which is fluorescently tagged and the other of which is not fluorescently tagged. The two probes are bound to each other. The unknown target in the sample is not fluorescently tagged. The presence or absence of the unknown target is determined by detecting competition, between the unknown target and the fluorescently tagged probe, for binding sites on the non-tagged probe. The fluorescently tagged probe can be a fluorescently tagged target or a fluorescently tagged probe that is known to competitively bind to the same binding site as the target. Alternatively, the presence or

absence of the unknown target is determined by detecting competition between the unknown target and the non-tagged probe for binding sites on the fluorescently tagged probe.

B. CROSSCORRELATION

Various system configurations can be used to study the binding properties of an unknown target using fluorescence correlation spectroscopy in crosscorrelation mode. The system is selected to enable the study of the coincidence of two fluorophores that emit radiation having maxima at two different wavelengths on the same complex using FCS. The two fluorophores of the system can be located on the various components of the system including an unknown target, one or more probes, a bead, and combinations thereof.

In one embodiment, the sample includes a number of unknown targets each of which includes a first fluorescent tag, and at least one probe that includes a second fluorescent tag. If a probe binds to an unknown target, then two fluorescent tags are present on the same complex, which causes the fluctuations detected at the two different detectors to be correlated.

In another embodiment, two fluorescently tagged probes capable of binding with two unique sites are added to a sample that includes unknown target. Each probe includes a different fluorescent tag and the coincidence of both fluorescent tags on an unknown target causes the fluctuations detected at the two different detectors to be correlated.

In another embodiment, the sample includes a number of unknown targets, each of which includes a common epitope and a unique binding site. A first fluorescent tag capable of binding to the epitope is added to the sample such that all of the unknown targets with the common epitope become labeled with the first fluorescent tag. The addition of a second fluorescently tagged probe and the binding of the second probe with an unique binding site on the unknown target results in the coincidence of two fluorescent tags on the same complex, which causes the fluctuations detected at the two different detectors to be correlated.

In another embodiment, the unknown target or a first probe includes a first fluorescent tag and the binding of the first probe to the unknown target results in the

creation of a new binding site to which a second probe is capable of binding. Addition of a second fluorescent tag in the form of a fluorescently tagged second probe and binding of the second probe to the newly created binding site results in the coincidence of two fluorescent tags on the same complex, which causes the fluctuations detected at the two different detectors to be correlated. Examples of useful methods by which a binding site is created have been described above.

In other embodiments, the probe, unknown target, fluorescent tag or a combination thereof is attached to a bead. In one embodiment, the probe is attached to a bead and at least one of the probe and the bead includes a first fluorescent tag. When the probe-bead complex is added to a sample and a fluorescently tagged unknown target binds to the probe, there is a coincidence of two fluorescent tags on the same complex, which causes the fluctuations detected at the two different detectors to be correlated.

In another embodiment, the unknown target is attached to a bead and at least one of the unknown target and the bead include a fluorescent tag. A fluorescently tagged probe is added to the sample and, if binding occurs between the probe and the unknown target, there is a coincidence of two fluorescent tags on the same complex, which causes the fluctuations detected at the two different detectors to be correlated.

In another embodiment, the probe is attached to a bead and at least one of the probe and the bead includes a first fluorescent tag. When an unknown target binds to the probe, the binding creates a site for a second probe to bind. Addition of a fluorescently tagged second probe and binding of the second probe to the newly created site on the unknown target causes a coincidence of two fluorescent tags on the same complex, which causes the fluctuations detected at the two different detectors to be correlated.

In another embodiment, the unknown target is attached to a bead and at least one of the unknown target and the bead includes a first fluorescent tag. When a probe binds to the unknown target, the binding creates a site for a second probe to bind. Addition of a fluorescently tagged second probe and binding of the second probe to the newly created site on the unknown target causes a coincidence of two fluorescent tags on the same complex, which causes the fluctuations detected at the two different detectors to be correlated.

The enzymatic reactions that add or remove moieties described above with respect to autocorrelation can also be used in crosscorrelation mode to create binding sites recognized by specific probes.

C. MULTIPLEXING

The complexity of the system and the detail of the information obtained from the system can be increased by including multiple bead sizes, multiple unique fluorophores, multiple unique probes, and combinations thereof. Systems that include multiple bead sizes, multiple unique fluorophores, multiple unique probes and combinations thereof can be analyzed according to autocorrelation, crosscorrelation and combinations thereof. The embodiments described above with respect to autocorrelation and crosscorrelation can all be modified to include multiple bead sizes, multiple unique fluorophores, multiple unique probes, and combinations thereof, which enables the simultaneous study of multiple molecular interactions.

In one embodiment, multiple unique probes are added to a sample of unknown targets to simultaneously determine the presence or absence of binding of one or more of the probes to the unknown targets. Each unique probe includes a unique fluorophore. Changes in the diffusion coefficients or particle numbers of one or more of the fluorescently tagged probes simultaneously provide information about the nature of the unknown targets in the sample. If no changes occur for any of the unique probes, for example, it can be determined that none of the unknown targets include any of the binding sites associated with the unique probes. Likewise if probe-unknown target binding occurs, the diffusion coefficient of the unique fluorophore(s) associated with the complex will change, which will provide the identity of the probe that has become bound to an unknown target, which in turn provides information about the nature of the unknown target. This information can be gained from each unique fluorescent tagged probe that exhibits a change in diffusion constant. In this embodiment, the FCS instrument used to measure the fluorescence includes a sufficient number of detectors to detect the unique emission wavelength emitted by each unique fluorophore. In other embodiments, the system that includes a number of unique fluorescent tags can be configured for crosscorrelation

analysis. Such systems configurations additionally include, e.g., unknown targets having the same fluorescent tag and beads having the same fluorescent tag.

If the unknown target is attached to a bead, the change in the diffusion coefficient of the fluorescently tagged probe that binds the unknown target can be more pronounced.

In another embodiment, multiple unique probes having different binding properties, are attached to beads. When the bead-probe complexes are added to a sample that includes a number of unknown targets, each of which includes a unique fluorescent tag, binding between a probe and an unknown target will be evidenced by a change in the diffusion coefficient for the fluorescently tagged target and can be detected at the detector associated with the emission wavelength of the fluorophore of the target. In other embodiments, the system can be configured for crosscorrelation analysis by including the same fluorescent tag on the probes or beads of the system.

In other embodiments, the sample includes a number of unique probes that include unique fluorescent tags and are capable of creating a new unique binding site when bound to a target. Introduction of a second probe that is capable of binding to the newly created site will cause a change in the diffusion coefficient of the unique fluorescent probes. The system optionally can be configured for crosscorrelation analysis by including the same fluorescent tag on at least one of the second probe and the unknown target.

In other embodiments, a number of different sized beads are added to the system. Each size bead has a known characteristic diffusion coefficient. Unique probes having unique binding characteristics are attached to each bead size such that each bead size has a unique binding characteristic associated with it. When the beads are added to a sample of fluorescently tagged unknown targets, a binding event is detected in an autocorrelation mode as a change in the diffusion coefficient of the fluorescently tagged unknown target. The new diffusion coefficient of the fluorescently tagged unknown target corresponds to the diffusion coefficient of a particular bead size, which in turn allows the determination of binding properties of the unknown target bound to the bead. In this embodiment, the fluorophores of the fluorescent tags can be the same. In another embodiment, at least one of the bead or the unique probes includes a unique fluorescent tag. When more than one unique fluorescent tag is present in the sample, the spectroscopy instrument includes a

sufficient number of detectors (i.e., detection channels) to detect the unique fluorescent tags in autocorrelation mode.

In another embodiment, the system includes different sized beads, which include unique fluorescent tags corresponding to the size of the bead, and unique probes attached to the beads. The system optionally can be configured for crosscorrelation analysis where the unknown targets include the same fluorescent tag. When more than one unique fluorescent tag is present in the sample, the spectroscopy instrument includes a sufficient number of detectors to detect the unique fluorescent tags in autocorrelation and crosscorrelation modes.

III. THE PROBE

The probe includes at least one binding site. Where multiple binding sites are present on a probe, the multiple binding sites can include, e.g., multiple binding sites for the same site of interest, multiple unique binding sites capable of binding unique sites of interest, and combinations thereof. The binding site of the probe and the properties of the probe can determine the specificity of the probe and the nature of the information that can be obtained when a binding event occurs with the probe.

Useful probes include, e.g., probes to which binding is desired, probes capable of binding to a site of interest, and combinations thereof.

Any suitable probe can be used. Suitable probes include, e.g., macromolecules (e.g., proteins, peptides, polynucleic acids, and polysaccharides), molecules (e.g., amino acids, nucleic acids, and saccharides), and combinations thereof. Useful macromolecules include, e.g., antibodies, receptor proteins, lectins, hormones, protein A, protein G, avidin, enzymes, and combinations thereof.

Suitable probes are commercially available from a variety of sources including, e.g., Sigma Biochemicals (St. Louis, Missouri), Molecular Probes (Eugene, Oregon), and Vector Laboratories (Burlingame, California).

The probe can optionally include a fluorescent tag. Useful fluorescent tags and methods of making the same are described below and incorporated herein.

The probe can optionally include a bead or other component that increases the mass of the probe. Useful methods of making the same are described below and

incorporated herein. Beads suitable for macromolecular and molecular attachment are commercially available from Sigma Biochemicals (St. Louis, Missouri), Molecular Probes (Eugene, Oregon), and Bangs Laboratories (Fishers, Indiana). Examples of useful mass adding components are described above and incorporated herein.

One useful method of determining the specificity of a probe, and whether the probe exhibits a suitable specificity for a binding site of interest, involves using FCS including, e.g., autocorrelation and crosscorrelation. The prepared probe is added to a sample that includes a target of interest, i.e., a target that includes a known binding site. The sample also includes a fluorescent tag and the components of the system are selected such that binding between the probe and the target corresponds to a detectable change in the diffusion coefficient of the fluorescent tag, change in particle number or a combination thereof. Measuring and analyzing the behavior of the known probe and its known target can verify specificity and selectivity of probes, i.e., if the probe binds to its target.

A. PROBES FOR PATHOGEN DETECTION

Various probes can be prepared for use in detecting pathogens. Antigens of the pathogens, for example, can be detected using specific antibody probes and nucleic acid sequences of the pathogen can be detected using specific oligonucleotide probes. The probes preferably are selected to target glycoproteins, proteins, nucleic acids, or combinations thereof, which may include specific portions of the pathogen, a spore of the pathogen, toxins, metabolic products of the pathogen, biological responses induced by the pathogen. The specific probe-sets can be prepared based on genomic data, the characterization of the expressed proteome, and clinical data on outcomes of infection.

Oligonucleotide probes can be designed based on information obtained using Polymerase Chain Reaction, PCR, and the analysis of genomic data for variable and conserved regions of DNA in related pathogen species. Antibody probes can be designed for unique antigens expressed by specific pathogens as determined by genomic and proteomic database analysis. Pathogenic virulence can often be traced to unique expression of one or more proteins or glycoproteins. Such unique expression of one or more proteins or glycoproteins can serve as a specific probe target.

Once selective probes have been identified and binding conditions optimized, combinations of probes can be selected to produce putative probe-sets. Suitable probe-sets include, e.g., two DNA specific probes, two antigen specific probes, and one DNA specific probe and one antigen specific probe.

For pathogen applications, a probe-set can be created to include at least two probes for each pathogen. The probes can be designed to detect the pathogen, toxins secreted by the pathogen and combinations thereof. For Bacillus anthracis, for example, suitable probes include probes capable of binding the protective antigen (PA) of anthrax, anthrax lethal factor (LF), pXO1 plasmid, pXO2 plasmid, and combinations thereof. Probes to PA or LF can detect products of Bacillus anthracis, and pXO1 and pXO2 probes can detect the presence of viable bacteria in the sample. Commercially available probes can be used as components of these probe-sets including, e.g. antibodies against B. anthracis, PA and LF.

Variola major virus simulant, vaccinia can be used to identify and test probe-sets for the detection and identification of viral pathogens. Probes can be designed to specifically bind target DNA sequences and target viral coat proteins. Polymerase Chain Reaction has been used to distinguish between variola and vaccinia virus, which indicates that specific oligonucleotide probes can be used to specifically bind unique viral DNA targets. The preferred vaccinia primer, 5'-ATG ACA CGA TTA CCA ATA-3' will be used as a probe to determine if vaccinia virus can be detected using FCS. A second primer (5'-CTA GAC TTT GTT CTC TG-3') which also binds to vaccinia DNA sequences will be used as a second DNA probe for FCS crosscorrelation analysis. Further genome analysis will be conducted to determine if other DNA sequences can serve as specific targets. Commercial antibodies are available for vaccinia virus, and will be tested for suitability as a FCS probe for viral coat proteins.

IV. THE TARGET

The target can include any target of interest. The target can be unknown or known. Unknown targets include those targets to which it is not known whether or not the probe binds. Known targets include those targets to which a binding site of a probe of interest binds. Examples of suitable targets include macromolecules (e.g., proteins, peptides, polynucleic acids, and polysaccharides), molecules (e.g., amino acids, nucleic acids, and

saccharides), and combinations thereof. Useful macromolecules include, e.g., antibodies, receptor proteins, lectins, hormones, protein A, protein G, avidin, enzymes, and combinations thereof.

The target can be a library, a portion of a library, a member of a library or a combination thereof. Suitable libraries include, e.g., apatamer libraries, phage display libraries, antibody libraries, peptide libraries, and translated cDNA libraries. Examples of members of a library include proteins, peptides, polynucleic acids, organic polymers, polysaccharides, amino acids, nucleic acids, and saccharides.

The target can optionally include a fluorescent tag. Useful fluorescent tags and methods of attaching fluorescent tags to components such as targets are described below and incorporated herein. The members of an expressed cDNA library can be labeled with a fluorescent tag during translation or post-translation. Suitable post-translational labeling methods include, e.g., modifying lysine amino groups or cysteine thiol groups with a reactive fluorescent moiety. Examples of amine reactive groups include, e.g., isothiocyanate, n-succidimidyl ester, and sulfonyl chloride. Examples of thiol reactive groups include acetamides and maleamides. Other post translational labeling methods are described, e.g., in G. T. Hermanson, Bioconjugate Techniques, 1996 (Academic Press, Inc., San Diego, California, pp. 785) and incorporated herein. Post translational labeling can target either endogenous sites on the protein or exogenous sites added for the specific purpose of targeting the fluorescent label.

In another method, the members of an expressed cDNA library can be labeled during an in vitro translation (IVT) method, i.e., incorporating a fluorescent label during the translation from cDNA to proteins. In one such method a fluorescent amino acid (e.g., fluorescently modified lysine) is incorporated into the proteins produced during translation. A useful example of a fluorophore for labeling lysine is bodipy. A useful bodipy labeled lysine is commercially available from ProMega (Madison, Wisconsin).

In another method, the members of an expressed cDNA library can be labeled at the in vitro translation step by incorporating the cDNA sequence for a fluorescent protein into the DNA clone. When the fluorescent cDNA sequence is subsequently translated, the expressed components will be fluorescently tagged.

In other methods, the members of the library can be modified to include a common epitope. The epitope is selected such that a probe is capable of binding to the epitope. Various methods of modifying a library such that the members include a common epitope tag are well known

Other suitable targets include organisms including e.g., pathogens (e.g., bacterial, viral, rickettsia), pathogen components, toxins, and macromolecules associated with an organism. Examples of pathogen components include pathogens, pathogen fragments, pathogen nucleic acids, pathogen proteins, pathogen carbohydrates, pathogen spores, pathogen toxins, metabolic products of pathogens, and combinations thereof.

Useful target organisms include, e.g., *Bacillus cereus, Bacillus subtilis*, various strains of non-pathogenic *E. coli*, and vaccinia virus.

Useful target pathogens include, e.g., *Bacillus anthracis* and *Variola major*.

Useful target toxins include, e.g., toxins of plant, insect, animal, pathogenic and non-pathogenic origin. Examples of a plant toxins include ricin toxin from *Ricinus communis*.

V. FLUORESCENT TAGS

The fluorescent tag includes a fluorophore and can be a fluorophore, fluorophore-containing moieties that are capable of binding to other moieties (e.g., fluorescently tagged probes and fluorescently tagged beads), and combinations thereof.

Examples of useful fluorophores include NBD (i.e., N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)), rhodamine, fluorescein, eosin, erythrosine, dansyl and acridine orange. Examples of suitable commercially available reactive fluorophores include, fluorescein isothiocyanate, tetramethylrhodamine isothiocyanate, fluorphores available under the BODIPY series of trade designations from Molecular Probes (Eugene, Oregon) including, e.g., BODIPY FL succinimidyl ester of 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, BODIPY R6G 4,4-difluoro-5-phenyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester, BODIPY TR-X, BODIPY 630/650-X, BODIPY 650/665-X, BODIPY FL Br_2 SE, BODIPY 500/510, BODIPY FL C_5, BODIPY FL SE, BODIPY FL SSE, BODIPY FL AEBSF, BODIPY FL-X, BODIPY FL-X SE, BODIPY FL CASE, BODIPY TMR-X SE, BODIPY 530/550, BODIPY 530/550 SE,

BODIPY 530/550 EDA, BODIPY R6G SE, BODIPY R6G-X SE, BODIPY 581/591 SE, BODIPY 576/589 SE, BODIPY 650/665-X SE, BODIPY 564/570, BODIPY 564/570 SE, BODIPY 493/503 SE, BODIPY 559/568 SE, BODIPY TR-X, SE, and BODIPY 630/650-X SE; the Cy series of trade designations including, e.g., Cy3.5 monofunctional NHS-ester, Cy5.5 monofunctional NHS-ester, Cy3 monofunctional NHS-ester, Cy5 monofunctional NHS-ester, and Cy7 monofunctional NHS-ester all of which are commercially available from Amersham Biosciences (Buckinghamshire, England); and the ALEXA FLUOR series of trade designations including, e.g., ALEXA FLUOR 488 carboxylic acid succinimidyl ester mixed isomers, ALEXA FLUOR 555 carboxylic acid succinimidyl ester, ALEXA FLUOR 647 carboxylic acid succinimidyl ester, ALEXA FLUOR 350, 405, 430, 500, 514, 532, 546, 555, 568, 594, 610, 633, 647, 660, 680, 700, and 750 all of which are commercially available from Molecular Probes (Eugene, Oregon).

Useful protocols for labeling proteins and other biomolecules with fluorophores can be found in, e.g., R. Haugland, Handbook of Fluorescent Probes and Research Products (Ninth Ed. 2002) and G. T. Hermanson, Bioconjugate Techniques (1996), and incorporated herein.

VI. CROSSLINKING AGENT

The system can optionally include a crosslinking agent. The crosslinking agent includes at least two binding sites and is capable of binding at least two components including, e.g., probe, target, and combinations thereof, to form a crosslinked structure. The component to which the crosslinking agent binds can optionally include multiple binding sites to which the crosslinking agent can bind. Crosslinking can manifest itself as an increase in the mass of the complex, a decrease in the number of particles and combinations thereof.

The crosslinking agent can function to aggregate the at least two components. Where at least one of the two components includes a fluorescent tag, the fluorescently tagged component exhibits an increase in mass, which can be detected as a change in the diffusion coefficient of the fluorescently tagged component. The aggregation of at least

two fluorescently tagged components can also be detected as a decrease in the number of independently moving fluorescently tagged components in the system.

At least one of the components of the system (e.g., probe, target, bead, and combinations thereof) can function as the crosslinking agent. Alternatively, the crosslinking agent can be an additional component of the system. Suitable crosslinking agents include, e.g., the probe, the target, a mass adding component, lectins against a component of the system, antibodies to a component of the system (e.g., the probe or the target), and combinations thereof.

VII. STOICHIOMETRY

Macromolecular stoichiometry, i.e., the quantitative proportions with which two macromolecules interact, of a target can be determined from autocorrelation data. Starting with Equation 4, macromolecular stoichiometry can be calculated if it is assumed that intersystem crossing and particle diffusion are independent sources of fluctuation. The first occurs in the microsecond (μs) time domain and results from intersystem crossing of fluorophores between the singlet and triplet states. The second occurs in the millisecond (ms) time domain, and results from the diffusion of particles into and out of the confocal volume. Fluctuations between singlet and triplet states are therefore governed by the number of the fluorophores in the confocal volume while diffusional fluctuations are governed by the number of particles. This leads to a simplified form of equation (4). If this assumption is made, Equation 4 can be rearranged to yield Equation 5

$$G(\tau) = 1 + \frac{T}{N_M}\exp(-\tau/\tau_T) + \frac{1-T}{N_P}\left(\sum_i \frac{F_i}{\left(1+\tau/\tau_{D_i}\right)\left(1+\tau/K^2\tau_{D_i}\right)^{1/2}}\right) \quad (5)$$

which can be further simplified to Equation 6

$$G(\tau) = 1 + \frac{1}{N'_M}\exp(-\tau/\tau_T) + \frac{1}{N'_P}\left(\sum_i \frac{F_i}{\left(1+\tau/\tau_{D_i}\right)\left(1+\tau/K^2\tau_{D_i}\right)^{1/2}}\right) \quad (6)$$

by defining $N_M = N'_M T$ and $N_P = N'_P(1-T)$.

One method of determining the molecule stoichiometry of a target employs the autocorrelation data and a calibration factor, r, which is the number of fluorophores per fluorescently tagged probe. The calibration factor, r, is calculated according to the following equation (7)

$$r = \frac{N'_M}{N'_P} \qquad (7)$$

where N'_M is the number of fluorophore molecules and N'_P is the total number of diffusing particles and includes slow diffusing complexes (F_2) (i.e., a complex of a fluorescently tagged probe bound to the target of the probe) and fast diffusing particles (F_1), (i.e., fluorescently tagged probes that are uncomplexed (i.e., unbound)) obtained from the autocorrelation data. Stoichiometry (S) is defined conceptually as

$$S = \frac{\text{Number of Fabs in slow complex}}{\text{Number of complexes}}$$

where S is given by

$$S = \frac{\text{Total Fabs} - \text{fast diffusing Fabs}}{\text{Number of complexes}}$$

such that the general relationship for stoichiometry can be written as

$$S = \frac{N'_M / r - F_1 N'_P}{F_2 N'_P} \qquad (8)$$

where $F_1N'_P$ defines the total number of fast diffusing particles, i.e., uncomplexed fluorescently tagged probes, and $F_2N'_P$ defines the total number of slow diffusing complexes formed. This analysis is carried out with tagged probe at constant concentration, and exposed to a range of target concentrations. S is obtained with Equation 8 for each target concentration using the parameter values obtained by fitting to Equation 6 and the calibration factor determined with Equation 7

VIII. NUMBER OF INDEPENDENTLY MOVING FLUORESCENT PARTICLES

A given detector detects the class of fluorescent tags in the sample that fluoresce at a given set of wavelengths. If the fluorescent tags become complexed as a result of molecular interactions (e.g., between probe, target, crosslinking agent and combinations

thereof) then the number of such independently moving fluorescent tags decreases. The number of such independently moving fluorescent tags, referred to herein as the particle number (N_p), present in the sample can be determined from the autocorrelation curve generated from the autocorrelation data. G(0), which is the value G(t) of the autocorrelation curve at time = 0, and which is inversely proportional to the number of particles (N_p) in the system. This value is typically calculated from the curve fit to the autocorrelation data since the actual 0 time point obtained experimentally is dominated by shot noise. The 0 time point of the autocorrelation function increases as the number of particles decreases. To determine the number of particles of interest in the confocal volume, the particle of interest must include a fluorescent tag. If the fluorescently tagged particles form an aggregate, the number of free fluorescently tagged particles will decrease resulting in a shift in the 0 time point of the autocorrelation function and a corresponding decrease in the number of particles in the system.

IX. THE CROSSCORRELATION CORRECTION ALGORITHM

Emission bleed-through between detectors occurs in FCS systems. Emission bleed-through occurs when detector configuration allows detection of both fluorophores in a single detector channel and leads to artifactual crosscorrelation of two signals. As a result, the measured autocorrelation and crosscorrelation functions differ from the true autocorrelation and crosscorrelation functions (i.e., those correlation functions that would be obtained in the absence of bleed through). Because of the high rates of data acquisition it is difficult to correct for bleed-through during acquisition. The present inventors have discovered that the three measured correlation functions (i.e., two autocorrelation functions and one crosscorrelation function) are linear combinations of the three true (i.e. bleed-through corrected) correlation functions. Correction for bleed-through becomes a matter of solving this set of three simultaneous linear equations.

To correct for this bleed through and to obtain the true autocorrelation and crosscorrelation functions, the autocorrelation and crosscorrelation data obtained for a sample is preferably further analyzed using algorithms that correct for crossover emission detected in the two detectors. For the autocorrelation function of a first detector, the data is preferably subjected to the following algorithm

$$G_{1T} = \frac{-2\rho < I_1 >< I_2 > R + \rho^2 < I_2 >^2 G_2 + < I_1 >^2 G_1}{-2\rho < I_1 >< I_2 > + \rho^2 < I_2 >^2 + < I_1 >^2} \quad (9)$$

where G_{1T} is the true autocorrelation function of the fluorescence measured at the first detector, ρ is the bleed through coefficient of detector two into detector one, $<I_1>$ is the time averaged intensity in detector one, $<I_2>$ is the time averaged intensity in detector two, R is the measured crosscorrelation function for detectors one and two, and G_1 and G_2 are the measured autocorrelation functions of detector one and detector two, respectively.

For the autocorrelation function of the second detector, the data is preferably subjected to the following algorithm

$$G_{2T} = \frac{-2r < I_1 >< I_2 > R + < I_2 >^2 G_2 + r^2 < I_1 >^2 G_1}{-2r < I_1 >< I_2 > + < I_2 >^2 + r^2 < I_1 >^2} \quad (10)$$

where G_{2T} is the true autocorrelation function of the fluorescence measured at the second detector, r is the bleed through coefficient of detector one into detector two, and R, $<I_1>$, $<I_2>$, G_1, and G_2 are as described above.

For the crosscorrelation function, the data is preferably subjected to the following algorithm

$$R_T = \frac{< I_1 >< I_2 > R(1 + \rho r) - \rho < I_2 >^2 G_2 - r < I_1 >^2 G_1}{< I_1 >< I_2 > (1 + \rho r) - \rho < I_2 >^2 - r < I_1 >^2} \quad (11)$$

where R_T is the true crosscorrelation function of the fluorescence measured at the first and second detectors, R is the measured crosscorrelation function of detector one and detector two, and r, ρ, $<I_1>$, $<I_2>$, G_1, and G_2 are as described above.

The bleed through coefficient, ρ, is experimentally determined by taking a first fluorophore and measuring its average intensity in detector two divided by its average intensity measured in detector one.

The bleed through of detector one into detector two, *r*, is experimentally determined by taking a second fluorophore and measuring its average intensity in detector one divided by its average intensity measured in detector two.

Equations (9)-(11) assume that there is no difference between the crosscorrelation of detector channel one with detector channel two, R_{12}, and the crosscorrelation of detector channel two with detector channel one, R_{21}. While this is both theoretically and practically true, one can, in fact determine R_{12} and R_{21} separately in which case equations (9)-(11) can be replaced with equivalent corresponding equations (9a), (10a), (11a) and (11b):

$$G_{1T} = \frac{-\rho < I_1 >< I_2 > (R_{12} + R_{21}) + \rho^2 < I_2 >^2 G_2 + < I_1 >^2 G_1}{-2\rho < I_1 >< I_2 > + \rho^2 < I_2 >^2 + < I_1 >^2} \quad (9a)$$

$$G_{2T} = \frac{-r < I_1 >< I_2 > (R_{12} + R_{21}) + < I_2 >^2 G_2 + r^2 < I_1 >^2 G_1}{-2r < I_1 >< I_2 > + < I_2 >^2 + r^2 < I_1 >^2} \quad (10a)$$

$$R_{12T} = \frac{< I_1 >< I_2 > (R_{12} + \rho r R_{21}) - \rho < I_2 >^2 G_2 - r < I_1 >^2 G_1}{< I_1 >< I_2 > (1 + \rho r) - \rho < I_2 >^2 - r < I_1 >^2} \quad (11a)$$

$$R_{21T} = \frac{< I_1 >< I_2 > (R_{21} + \rho r R_{12}) - \rho < I_2 >^2 G_2 - r < I_1 >^2 G_1}{< I_1 >< I_2 > (1 + \rho r) - \rho < I_2 >^2 - r < I_1 >^2} \quad (11b).$$

In another embodiment, the bleed through emission can be corrected for on the measured signals at the detector channels (i.e., detector channels one and two). The true signals X and Y for detector channel one and two, respectively is obtained by measuring the signals I_1 and I_2 for detector channels one and two, determining the bleed through coefficient, ρ, i.e., the fraction of the signal in detector channel one due to the signals from detector channel two, determining the bleed through r, i.e., fraction of the signal in detector channel two that is due to the signals in detector channel one, correcting the signal in channel one using the equation

$$\boxed{X = \frac{I_1 - \rho I_2}{1 - \rho r}}$$

and optionally, additionally, or alternatively correcting the signal in channel two using the equation

$$\boxed{Y = \frac{I_2 - rI_1}{1 - \rho r}}.$$

X. OTHER PROPERTIES OF THE SYSTEM

A. FLOW

The sample can be provided in the confocal volume of the FCS instrument in a variety of forms including, e.g., a well, cuvette, flow chamber, and capillary tube. In the case of a flow chamber, the sample flows through the confocal volume. The flow can be the result of a variety of forces including, e.g., pressure (e.g., a hydrostatic flow where the velocity of the particle is independent of the size of the particle), and applied voltage, e.g., electrophoretic flow, where the velocity of the particle is dependent on the size of the particle.

B. HIGH THROUGHPUT AND AUTOMATED SYSTEMS

In another aspect, high throughput screening methodologies, such as screening libraries by selection of subvolumes can be utilized to identify probe-member binding pairs, i.e., binding events between a probe and member of a library. For example, a stock of library members can be divided into subvolumes such that each subvolume contains a portion of the members of the library. Each subvolume solution is then screened utilizing an array (e.g., multiple sample chambers containing members of the library (i.e., potential targets)). Upon detection of a binding event, the subvolume can be sub-divided again and screened repeatedly until the member that binds to the probe is identified.

The system can also be automated. Suitable automated systems include those robotic systems developed for solution phase chemistries. These automated systems include automated workstations including, e.g., the automated synthesis apparatus developed by Takeda Chemical Industries, LTD. (Osaka, Japan) and the many robotic systems that utilize robotic arms (Zymate II, Zymark Corporation, Hopkinton, Mass.; Orca, Hewlett-Packard, Palo Alto, Calif.). Suitable automated systems include, e.g., providing (e.g., sequentially or simultaneously) multiple samples to a sample detection

volume of an FCS instrument. Suitable automated systems also include automated liquid handling including, e.g., including probes, buffers, targets, fluorescent tags and combinations thereof.

C. THE SAMPLE

The sample can be obtained from a variety of sources including, e.g., samples obtained by swabbing (e.g., cheek swab, nose swab, and eye swab), biological samples including, e.g., bodily fluids (e.g., blood, urine, saliva, and ear wax), environmental samples including, e.g., water, air, and soil, and combinations thereof.

XI. KITS

The reagents of the system including, e.g., at least one probe and at least one fluorescent tag, can be included in a kit for assaying for the presence of an unknown or a known target. The probes can be capable of binding to a predetermined target or site on a macromolecule. The fluorescent tag can be attached to the probe. The kit can optionally include a bead or a plurality of beads. At least one probe can be attached to the bead. The bead can optionally include a fluorescent tag. The kits can include multiple fluorescent tags having unique fluorophores.

One useful kit includes fluorescently tagged human serum albumin galactose probe. Another useful kit includes a human serum albumin galactose probe and a fluorescently labeled ricin probe. Such kits are useful for assaying for toxin including, e.g., ricin.

The invention will now be described by way of the following examples.

EXAMPLES

Test Procedures

Test procedures used in the examples include the following.

FCS Instrumentation

The samples were analyzed using a fluorescence correlation spectroscopy instrument configured to distinct fluorescence emission at different wavelengths and a data

processing program capable of autocorrelation, crosscorrelation, Fourier transform and Moment analyses.

EXAMPLE 1

Bacteria of an unknown strain were tagged nonspecifically with a lipophilic fluorescent dye, DiIC16. A specific antibody for E. coli strain K-12 was tagged with ALEXA-546. The tagged antibody is not specific for the bacteria of the unknown strain and therefore does not bind to it. The fluorescently tagged bacteria and the fluorescently tagged antibody for E. coli were measured in two unique detection channels (i.e., channels 1 and 2) tuned to a wavelength for detecting ALEXA-546 and DiIC16, respectively. There were no coincident peaks in detection channels 1 and 2 and crosscorrelation did not occur. FIG. 1A illustrates the absences of coincident peaks in detection channels 1 and 2. The peaks in detection channel 2 represent two bacteria moving through the confocal detection volume. The lack of coincident peaks in detection channel 1 suggests that the ALEXA-546 tagged antibody for E. coli does not bind to the fluorescently tagged bacterial strain. FIG. 1B illustrates the absence of a positive crosscorrelation curve due to the lack of coincident peaks in detection channels 1 and 2.

EXAMPLE 2

E. coli strain K-12 tagged with ALEXA-594 (sold under the trade designation BIOPARTICLES, Product# D23370) were purchased from Molecular Probes (Eugene, Oregon). An E. coli specific antibody that was conjugated to ALEXA-546 and that specifically recognizes binding sites on the K-12 strain of E. coli was used as a second probe. The antibody was incubated with *E. coli* for 5 minutes at room temperature so as to achieve equilibrium binding. The ALEXA-594 and ALEXA 546 were measured in unique detection channels 1 and 2, which were tuned to a wavelength for detecting ALEXA-594 and ALEXA-546, respectively, and when signal peaks were coincident between both detection channels, a crosscorrelation curve resulted. FIG. 2A illustrates a single coincident peak at detection channels 1 and 2, which represents the movement of a single bacterium through the confocal detection volume. FIG. 2B illustrates the crosscorrelation curve that results from coincident peaks shown in FIG. 2A.

EXAMPLE 3

E. coli strain K-12 tagged with ALEXA-594 (sold under the trade designation BIOPARTICLES, Product# D23370) were purchased from Molecular Probes (Eugene, Oregon). An E. coli specific antibody that had been conjugated to ALEXA-546 and that specifically recognizes binding sites on the K-12 strain of E. coli was used as a second probe. The antibody was incubated with *E. coli* for 5 minutes at room temperature so as to achieve equilibrium binding. The ALEXA-594 and ALEXA 546 were measured in two unique detection channels 1 and 2, which were tuned to a wavelength for detecting ALEXA-594 and ALEXA-546, respectively. When signal peaks were coincident between both detection channels, a crosscorrelation curve resulted. FIG. 3A illustrates two coincident peaks at detection channels 1 and 2, which represent two bacteria moving through the confocal detection volume. FIG. 3B illustrates the crosscorrelation curve that results from coincident peaks shown in FIG. 3A.

EXAMPLE 4

Antibodies for E. coli were tagged with ALEXA-546 and added to a sample chamber that included E. coli and incubated for 5 minutes to achieve equilibrium. Autocorrelation data were collected for the sample. FIG. 4A illustrates two peaks, which represent individual bacteria moving through the detection volume. FIG. 4B illustrates the autocorrelation curve for the data collected in FIG. 4A.

EXAMPLE 5

Fab fragments of whole antibody were tagged with Rhodamine (Rh-Fab). Rh-Fab binds specifically to antibody (IgG) but the number of specific binding sites was not known. To determine the number of binding sites on IgG for Rh-Fab, 10 nM of Rh-Fab was titrated with increasing concentration of IgG and allowed to equilibrate. FIG. 5A illustrates the autocorrelation curves of Rh-Fab alone (20) and in the presence of 4 μM IgG (24). Data were fit to Equation 6 and best fit regression lines and residuals (top panel) displayed. FIG. 5B illustrates the use of the parameter estimates obtained from this analysis to determine the fraction of slow diffusing particles (F_2Np) at each IgG

concentration. The number of slow diffusing particles increases as IgG increased, and saturated when all binding sites were occupied. FIG. 5C illustrates the use of paramenter estimates from the analysis of autocorrelation curves (FIG. 5A) in Equations 7 and 8 to determine stoichiometry of binding Rh-Fab to IgG. The number of Rh-Fab bound to IgG was determined using Equation 9, and plotted for each IgG concentration tested. This analysis shows that there are about 6 Rh-Fab bound to each IgG at low IgG concentrations, and about 2 Rh-Fab bound at high IgG concentrations.

EXAMPLE 6

The low affinity nerve growth factor receptor, gp75, which binds to nerve growth factor (NGF) were expressed endogenously in A875 cells. A875 cells were placed in a cell culture chamber overnight and allowed to attach to the surface of the chamber. Cell were either left untreated, or exposed to 180 nM of NGF for 5 minutes to reach binding equilibrium. FIG. 6A illustrates the autocorrelation curves from untreated (30) and NGF-treated (34) A875 cells. Data were fit to Equation 6 and best fit regression lines and residuals (top panel) displayed. FIG. 6B illustrates theoretical curves for monomers 36, dimmers 38, trimers 40 and tetramers 42 as a function of fractional occupancy. The parameter estimates obtained from this analysis were used in Equation 8 to determine that the results of the stoichiometry calculations for untreated (1.12) and NGF-treated (0.94) were not different, which suggests that gp75 are receptor monomers in A875 cells.

EXAMPLE 7

Ricin was fluorescently labeled with ALEXA-546 to facilitate assay development. FIG. 7 illustrates ALEXA-546 conjugated ricin alone (50), in the presence of anti-ricin antibody (54), and in the presence of anti-ricin antibody that has been pre-incubated with unlabeled ricin (58) for 5 minutes at room temperature to reach equilibrium. When ALEXA-546 conjugated ricin binds to the anti-ricin antibody, there is a decrease in number of free particles (N) and a rightward shift in the diffusion time, indicating a slower diffusing (larger) particle. When anti-ricin antibody is preincubated with unlabeled ricin, the ricin binding sites are blocked, which prevents binding of ALEXA-546 conjugated ricin to the ricin antibody.

EXAMPLE 8

FIG. 8 illustrates Ricin tagged with ALEXA-546 (Al546-ricin) and prebound to galactose conjugated to Human Serum Albumin (HSA-gal) (60) for at least 5 minutes at room temperature to achieve equilibrium. Untagged ricin was added and incubated for 15 minutes at room temperature to achieve equilibrium displacement of Al546-ricin from HSA-galactose and a shift in the autocorrelation curve (64).

EXAMPLE 9

FIG. 9 illustrates the crosscorrelation data collected on bacteria particles labeled with both ALEXA-594 and ALEXA-546. The top panel (FIG. 9A) illustrates the crosscorrelation data as it appears before applying the cross-talk correction algorithm. The bottom panel (FIG. 9B) illustrates the same data after applying the correction algorithm (Equation 11). The slower diffusion time, which represents the bacterial particle diffusion, fits to 6 ms for both data sets.

EXAMPLE 10

FIG. 10 illustrates correction of autocorrelation data using the correction algorithm on bacteria particles labeled with both ALEXA-594 and ALEXA-546. FIG. 10A illustrates the autocorrelation data as it appears before applying the cross-talk correction algorithm. FIG. 10B illustrates the same data after applying the correction algorithm (Equation 9, for detector channel 1). The same analysis can be applied to data collected in detector channel 2 using Equation 10.

EXAMPLE 11

FIG. 11 illustrates Moment analysis of the fluorescence intensity fluctuations of ALEXA-546 tagged ricin either alone or bound to HSA-Galactose (data of Example 8). Moment analysis allows us to obtain a value for the particle number (N_P) without having to calculate the autocorrelation function. The data collected is subjected to the following general algorithm

$$\frac{< I^2 > - < I >^2}{< I >^2} \quad (12)$$

where I is the fluorescence intensity for the autocorrelation data being analyzed, <I> is the time averaged intensity (i.e. the first moment), and $<I^2>$ is the time averaged intensity squared (i.e. the second moment. Moment analysis of ricin alone resulted in a value of $1/N_P = 0.00014$. Moment analysis of ricin bound to HSA-Galactose resulted in a value of $1/N_P = 0.007$. FIG. 11 illustrates the analysis of the autocorrelation function of ricin alone (B) and ricin bound to HSA-Galactose (A). The arrows indicate $1 + 1/N_P$ calculated by Moment analysis.

EXAMPLE 12

FIG. 12 illustrates Fourier transform analysis of the fluorescence intensity fluctuations of ALEXA-546 tagged ricin alone (60) and bound to HSA-Galactose (64) (data of Example 8). FIG. 12A shows the power spectrum of the Fourier Transform (FFT function, Origin 5.0, OriginLab, Northampton, Massachusetts). The data collected is subjected to the following general algorithm.

$$\Im\left(\frac{I_i - < I >}{< I >}\right) \quad (13)$$

where I_i is the intensity value at each time point and <I> is the time averaged intensity. FIG. 12B shows the amplitude of the Fourier Transform.

Other embodiments are within the claims.

What is claimed is: